REMARKS/ARGUMENTS

In response to the Office Action of May 28, 2004, Applicants request re-examination and reconsideration of this application for patent pursuant to 35 U.S.C. 132.

Support for Amendments/Claim Status

No new matter has been added by the amendment to the specification made herein.

The disclosure of prior art, PCT/EP97/04396, at page 5 has been amended to correct a typographical error in the international application number. The corresponding international publication number has also been added.

No new matter has been added by the amendments to the claims made herein.

Claims 1, 39 and 44-46 have been amended. Claims 2-38 were cancelled in a previous reply (March 12, 2004). Claims 39-46 are withdrawn from consideration. Claim 1 is under examination. Claims 1 and 39-46 remain pending in the instant application.

Claim 1 has been amended to specifically claim the biopolymer marker (amino acid residues 2-14 of SEQ ID NO:1). The term "biopolymer marker" is used throughout the originally filed specification, see, for example, page 1, line 8.

Claim 39 has been amended to more clearly disclose the relationship between the presence of the claimed biopolymer marker (amino acid residues 2-14 of SEQ ID NO:1) and Alzheimer's disease.

Claim 39 has also been amended to clearly indicate how the presence of the claimed biopolymer marker is determined from mass spectrum profiles. The changes to claim 39 find basis through out the original disclosure, see, for example, page 35, lines 14-18, page 46, lines 10-19 and Figure 2.

Claim 44 has been amended to correspond with the biopolymer marker of claim 1 (as amended herein). Support for various types of kits can be found in the original disclosure, see, for example, page 36, lines 9-12 and page 47, line 15 to page 49, line 1.

Claims 45 and 46 were amended to provide proper antecedent basis for the term "kit" in claim 44 (as amended herein).

Restriction

The Examiner has indicated that newly submitted claims 39-46 (submitted March 12, 2004) are directed to an invention that is independent or distinct from the invention originally claimed for the following reasons: claims 39-43 are directed to methods of diagnosing Alzheimer's disease and claims 44-46 are drawn to kits. The Examiner states that since Applicants have received an action on the merits for the originally presented invention, the invention has been constructively elected by original presentation for prosecution on the merits. Thus, the Examiner has withdrawn claims 39-46 from consideration as being drawn to a non-elected invention.

Request for Rejoining of Claims

Considering that claims 39-46 are limited to the use of an isolated biopolymer marker consisting of amino acid residues 2-14 of SEQ ID NO:1, a search of these claims would encompass this specific biopolymer marker. The instant application is related in claim format to several other applications, both pending and issued, of which serial number 09/846,352 is exemplary. In an effort to maintain equivalent scope in all of these applications, Applicants respectfully request that the Examiner consider rejoining claims 39-46 in the instant application, which are currently drawn to non-elected inventions, under the decision in *In re Ochiai* (MPEP 2116.01) with claim 1 of the elected invention, upon the Examiner's determination that the claim of the elected invention is allowable and in light of the overlapping search. If the biopolymer marker consisting of amino acid residues 2-14 of SEQ ID NO:1 is found to be novel, methods and kits limited to its use should also be found novel.

Interview with the Examiner

Applicants thank the Examiner for courtesies extended to Applicants' representatives in the telephonic interview on October 28, 2004.

Applicants' representatives requested this interview in order to discuss how to proceed with filing of an RCE in the instant application.

In this interview, the Examiner stated that claim 1, as presented in the proposed amendment of September 2, 2004, would be rejected under 35 USC 112, second paragraph for the recitation of "related to Alzheimer's disease". The proposed claim 1 would also be rejected 35 USC 101, for lack of a specific and substantial utility and under 35 USC 112, first paragraph for lack of enablement and not disclosing how to use the claimed peptide. The Examiner also stated that major issues to support the utility rejection have already been presented in the previous Office Actions of record.

The state of the art of diagnostics of Alzheimer's disease was also discussed.

Rejection under 35 USC 112

Claim 1, as presented on March 12, 2004, stands rejected under 35 USC 112, first paragraph, as containing subject matter which allegedly was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

The Examiner continues to maintain the position that the instant specification, as filed, fails to provide any evidence or sound scientific reasoning that would support a conclusion that the isolated peptide consisting of amino acid residues 2-14 of SEQ ID NO:1 could be used in the diagnosis of Alzheimer's disease.

Applicants respectfully disagree with the Examiner's position.

The Examiner continues to assert that Applicant presented arguments that were fully answered in the previous Office Actions of record.

Applicants respectfully disagree with the Examiner's assertions. The arguments presented were responsive to the Examiner's previous Office Action.

Although Applicants believe that the instant specification fully supports the claim that an isolated peptide consisting of amino acid residues 2-14 of SEQ ID NO:1 is diagnostic for Alzheimer's disease, in the interest of compact, efficient prosecution Applicants have amended the claims to recite that the isolated peptide is linked to Alzheimer's disease.

According to the web site dictionary.com the term "linked" refers to the condition of being associated with or connected to (see attached document as accessed from the internet). The instant specification fully supports a connection and/or an association of the claimed peptide with Alzheimer's disease. The instant specification states at page 35, lines 14-18 that an objective of the invention is to evaluate samples containing a plurality of biopolymers for the presence of disease specific marker sequences which evidence a link to at least one specific disease state.

Claim 1 has been amended to specifically recite an isolated peptide consisting of amino acid residues 2-14 of SEQ ID NO:1. Claim 1, as amended herein, does not recite that the claimed

isolated peptide is diagnostic for Alzheimer's disease, nor does it recite that the claimed isolated peptide is related to Alzheimer's disease, even though Applicants believe that the specification as originally filed fully supports both of these recitations. Furthermore, the phrase "consisting of" is closed language and excludes any element, step or ingredient not specified in the claim (see MPEP 2111.03) Thus, the scope of claim 1 is limited to this specific peptide.

All questions of enablement are evaluated against the claimed subject matter (MPEP 2164.08); thus Applicants are not required to enable material which is not claimed. Further, it is well-established that claims are interpreted in light of the specification. Thus, in the instant case, the specification should enable the evaluation of samples containing a plurality of biopolymers for the presence of disease specific marker sequences which evidence a link to at least one specific disease state; the marker sequence being amino acid residues 2-14 of SEQ ID NO:1 which evidences a link to Alzheimer's disease. All that is necessary is that one skilled in the art be able to practice the claimed invention, given the level of knowledge and skill in the art (MPEP 2164.08).

Applicants assert that the skill in the art is high and it is obvious that no undue experimentation would be required for a skilled artisan to follow any of the electrophoretic, chromatographic and mass spectrometric protocols presented in the

instant specification in order to use the claimed invention. The Examiner fully agrees with Applicants assertion (page 4, final Office Action mailed on May 28, 2004). One of skill in the art would be able to view a gel, such as that shown in Figure 1 from which the claimed peptide was identified, and recognize a difference between two comparable samples (disease-state vs. non-disease state) and further recognize that the peptides present within the gel are differentially expressed between the two samples.

However, the Examiner maintains that the association of the claimed peptide with Alzheimer's disease is not supported by any evidence of record. Applicants respectfully disagree with the Examiner and contend that one of skill in the art would understand that the claimed peptide is linked to Alzheimer's disease.

When a peptide is identified in a body fluid sample from an Alzheimer's patient, it is immediately recognized as a potential diagnostic marker, even if the involvement of the peptide in the pathology of Alzheimer's disease is unknown. One of skill in the art would be familiar with this practice since it has been known in the art since at least 1992. See attached abstract of Gunnersen et al. (Proceedings of the National Academy of Science USA 89(24):11949-11953 1992) in which the detection of glutamine synthetase in the cerebrospinal fluid of Alzheimer's disease patients lead to the suggestion of glutamine synthetase as a potential diagnostic biochemical marker. Thus, when one of skill in

the art observes the claimed peptide identified in samples from Alzheimer's patient or differentially expressed between Alzheimer's disease patients and non-diseased patients; one of skill in the art would connect this peptide with potential diagnostics and/or therapeutics for Alzheimer's disease.

The claimed peptide is identified as a fragment of complement C3 precursor protein at page 46, lines 10-13 of the instant specification. It is widely believed that the activated complement system is responsible for many of the cellular changes involved in Alzheimer's disease: see attached abstracts; McGeer et al. Neuroscience Letters 107:341-346 1989; Veerhuis et al. Virchows Arch. 426(6):603-610 1995; and Eikelenboom et al. Neurobiology of Aging 17(5):673-680 1996 and article, Bradt et al. Journal of Experimental Medicine 188(3):431-438 1998. Thus, an ordinary skilled artisan would find it entirely reasonable to expect that the claimed peptide is linked to Alzheimer's disease.

Additionally, the Examiner asserts that a definitive diagnosis of Alzheimer's disease could only be made during postmortem examination or at brain biopsy. The mere fact that something has not previously been done clearly is not, in itself, a sufficient basis for rejecting all applications purporting to disclose how to do it. Diagnostic methods other than postmortem examination and brain biopsy have been deemed valuable for diagnosing Alzheimer's disease. For example, Applicants submit their own patent, US 6,451,547 B1 (Jackowski et al.) which claims methods for diagnosing

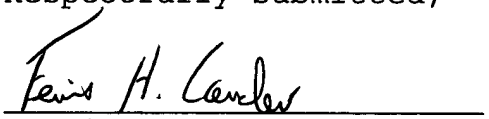
Alzheimer's disease by detecting the presence of biochemical markers in bodily fluid.

In conclusion, Applicants claim that the presence of a marker consisting of amino acid residues 2-14 of SEQ ID NO:1 is indicative of a link to Alzheimer's disease; a statement which is enabled by the instant specification, as shown by the arguments presented herein. Applicants assert that one of ordinary skill in the art when reviewing the instant specification, given the level of knowledge and skill in the art, would recognize how to use the claimed peptide as a marker for Alzheimer's disease. Thus, Applicants respectfully request that this rejection under 35 USC 112, first paragraph now be withdrawn.

CONCLUSION

In light of the foregoing remarks, amendment to the specification and amendments to the claims, it is respectfully submitted that the Examiner will now find the claims of the application allowable. Favorable reconsideration of the application is courteously requested.

Respectfully submitted,



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link[1] Pronunciation Key (lĭngk)
n.

1. One of the rings or loops forming a chain.
2. a. A unit in a connected series of units: *links of sausage; one link in a molecular chain.*
   b. A unit in a transportation or communications system.
   c. A connecting element; a tie or bond: *grandparents, our link with the past.*
3. a. An association; a relationship: *The Alumnae Association is my link to the school's present administration.*
   b. A causal, parallel, or reciprocal relationship; a correlation: *Researchers have detected a link between smoking and heart disease.*
4. A cuff link.
5. *Abbr.* **li** A unit of length used in surveying, equal to 0.01 chain, 7.92 inches, or about 20.12 centimeters.
6. A rod or lever transmitting motion in a machine.
7. *Computer Science.* A segment of text or a graphical item that serves as a cross-reference between parts of a hypertext document or between files or hypertext documents. Also called **hotlink, hyperlink**.

v. **linked, link·ing, links**
v. tr.

1. To connect with or as if with a link: *linked the rings to form a chain.* See Synonyms at join.
2. *Computer Science.* To make a hypertext link in: *linked her webpage to her employer's homepage.*

v. intr.

1. To become connected with or as if with a link: *The molecules linked to form a polymer.*
2. *Computer Science.* To follow a hypertext link: *With a click of the mouse, I linked to the company's website.*

[Middle English `linke`, *of Scandinavian origin*; akin to Old Norse `hlekkr, *hlenkr`, from *`hlenkr`.]

link'er *n.*

linked Pronunciation Key (lĭngkt)
adj.

1. Connected, especially by or as if by links.
2. *Genetics.* Exhibiting linkage.
3. *Computer Science.* Provided with links.

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linked (lĭngkt)
adj.

Exhibiting linkage.

Source: *The American Heritage® Stedman's Medical Dictionary*

Main Entry: **linked**
Pronunciation: `'li[ng](k)t`
Function: *adjective*
: marked by linkage and especially genetic linkage <*linked* genes>

Source: *Merriam-Webster Medical Dictionary, © 2002 Merriam-Webster, Inc.*

linked

Link \Link\ (l[i^][ng]k), v. t. [imp. & p. p. Linked (l[i^][ng]kt); p. pr. & vb. n. Linking.] To connect or unite with a link or as with a link; to join; to attach; to unite; to couple.

All the tribes and nations that composed it [the Roman Empire] were linked together, not only by the same laws and the same government, but by all the facilities of commodious intercourse, and of frequent communication. --Eustace.

Source: *Webster's Revised Unabridged Dictionary, © 1996, 1998 MICRA, Inc.*

linked

adj : connected by a link, as railway cars or trailer trucks [syn: coupled, joined]

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linked

linked: in CancerWEB's On-line Medical Dictionary

Source: *On-line Medical Dictionary, © 1997-98 Academic Medical Publishing & CancerWEB*

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- Thesaurus.com - Search for synonyms and antonyms

1: Neurosci Lett. 1989 Dec 15;107(1-3):341-6.

Activation of the classical complement pathway in brain tissue of Alzheimer patients.

McGeer PL, Akiyama H, Itagaki S, McGeer EG.

University of British Columbia, Kinsmen Laboratory of Neurological Research, Vancouver, Canada.

Positive immunohistochemical staining of Alzheimer brain tissue was obtained with antibodies to proteins associated with classical, but not the alternative, complement pathway. Clq, C3d, C4d are fractions of complement proteins that bind to tissue when the classical complement pathway is activated. Antibodies to these fractions stained senile plaques, dystrophic neurites and some neurofibrillary tangles. C5b-9 is the membrane attack complex which promotes cell lysis when assembled on the plasma membrane. An antibody to a neoantigenic site on this complex stained dystrophic neurites and many neurofibrillary tangles, but not extracellular amyloid. Properdin and fraction Bb of factor B, two proteins that bind to tissue when the alternative complement pathway is activated, were not detected immunohistochemically.

PMID: 2559373 [PubMed - indexed for MEDLINE]

2132.090

1: Proc Natl Acad Sci U S A. 1992 Dec 15;89(24):11949-53.

Related Articles, Links

Detection of glutamine synthetase in the cerebrospinal fluid of Alzheimer diseased patients: a potential diagnostic biochemical marker.

Gunnersen D, Haley B.

Department of Biochemistry, College of Pharmacy, University of Kentucky, Lexington 40536-0084.

In this report, 8- and 2-azidoadenosine 5'-[gamma-32P]triphosphate were used to examine cerebrospinal fluid (CSF) samples for the presence of an ATP binding protein unique to individuals with Alzheimer disease (AD). A 42-kDa ATP binding protein was found in the CSF of AD patients that is not observed in CSF from normal patients or other neurological controls. The photolabeling is saturated with 30 microM 2-azidoadenosine 5'-[gamma-32P]triphosphate. Photoinsertion can be totally prevented by the addition of 25 microM ATP. Photoinsertion of 2-azidoadenosine 5'-triphosphate into the protein is only weakly protected by other nucleotides such as ADP and GTP, indicating that this is a specific ATP binding protein. A total of 83 CSF samples were examined in a blind manner. The 42-kDa protein was detected in 38 of 39 AD CSF samples and in only 1 of 44 control samples. This protein was identified as glutamine synthetase [GS; glutamate-ammonia ligase; L-glutamate:ammonia ligase (ADP-forming), EC 6.3.1.2] based on similar nucleotide binding properties, comigration on two-dimensional gels, reaction with a polyclonal anti-GS antibody, and the presence of significant GS enzyme activity in AD CSF. In brain, GS plays a key role in elimination of free ammonia and also converts the neurotransmitter and excitotoxic amino acid glutamate to glutamine, which is not neurotoxic. The involvement of GS, if any, in the onset of AD is unknown. However, the presence of GS in the CSF of terminal AD patients suggests that this enzyme may be a useful diagnostic marker and that further study is warranted to determine any possible role for glutamate metabolism in the pathology of AD.

PMID: 1361232 [PubMed - indexed for MEDLINE]

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2132.090

1: Virchows Arch. 1995;426(6):603-10.

Complement activation in amyloid plaques in Alzheimer's disease brains does not proceed further than C3.

Veerhuis R, van der Valk P, Janssen I, Zhan SS, Van Nostrand WE, Eikelenboom P.

Department of Neuropathology, Free University Hospital, Amsterdam, The Netherlands.

In Alzheimer's disease (AD) patients, the complement components Clq, C4 and C3 can be detected in different types of beta/A4 plaques, one of the hallmarks of AD. Contradictory findings on the presence of late complement components in AD brains have been reported. Nevertheless, it was suggested in recent studies that in AD brain complement activation results in complement membrane attack complex (MAC) formation and that complement activation may act as an intermediate between beta/A4 deposits and the neurotoxicity observed in AD. In the present study the presence of a number of complement components and regulatory proteins in AD temporal cortex and, for comparison, in glomerulonephritis (GN) was analysed. In GN kidneys, besides Clq, Clr, Cls and C3, the late components and the C5b-9 complex are also associated with capillary basement membrane and mesangial immune complex deposits. In AD temporal cortex Clq, C4 and C3 are co-localized with beta/A4 deposits. However, in contrast to the GN kidney, the late complement components C5, C7 and C9, as well as the C5b-9 membrane attack complex cannot be detected in beta/A4 positive plaques. The absence of the cytolytic C5b-9 complex in AD brain suggests that in AD, the complement MAC does not function as the proposed inflammatory mediator between beta/A4 deposits and the neurofibrillary changes.

PMID: 7655742 [PubMed - indexed for MEDLINE]

2132.090

Eikelenboom P, Veerhuis R. The role of complement and activated microglia in the pathogenesis of Alzheimer's disease. Neurobiology of Aging. 17(5):673-680, 1996.

Abstract

A variety of inflammatory mediators including complement activation products, protease inhibitors, and cytokines are colocalized with β-amyloid (Aβ) deposits in the Alzheimer's disease (AD) brain. Activation products of the early complement components C1, C4, and C3 are always found in neuritic plaques and to a lesser extent in varying numbers of diffuse plaques. In contrast to these findings, no immunohistochemical evidence was obtained for the presence of the late complement components C7 and C9 and the complement membrane attack complex in the neuropathological lesions in AD brains. The mRNA encoding the late complement components C7 and C9 appears to be hardly or not detectable. These findings indicate that in AD the complement system does not act as an inflammatory meditor through membrane attack complex formation, but through the actions of the early complement products. In this review we focus on the role of complement in the pathological amyloid cascade in AD. In our opinion, the early complement activation products play a crucial role as mediators between the Aβ deposits and the inflammatory responses leading to neurotoxicity. .

Return to Complement References.

Complement-dependent Proinflammatory Properties of the Alzheimer's Disease β-Peptide

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*From the *Department of Immunology, The Scripps Research Institute, La Jolla, California 92037; and ‡Advanced Research Technologies, Inc., San Diego, California 92121*

Summary

Large numbers of neuritic plaques (NP), largely composed of a fibrillar insoluble form of the β-amyloid peptide (Aβ), are found in the hippocampus and neocortex of Alzheimer's disease (AD) patients in association with damaged neuronal processes, increased numbers of activated astrocytes and microglia, and several proteins including the components of the proinflammatory complement system. These studies address the hypothesis that the activated complement system mediates the cellular changes that surround fibrillar Aβ deposits in NP. We report that Aβ peptides directly and independently activate the alternative complement pathway as well as the classical complement pathway; trigger the formation of covalent, ester-linked complexes of Aβ with activation products of the third complement component (C3); generate the cytokine-like C5a complement-activation fragment; and mediate formation of the proinflammatory C5b-9 membrane attack complex, in functionally active form able to insert into and permeabilize the membrane of neuronal precursor cells. These findings provide inflammation-based mechanisms to account for the presence of complement components in NP in association with damaged neurons and increased numbers of activated glial cells, and they have potential implications for the therapy of AD.

Key words: Alzheimer's disease • amyloid • C3 • complement • inflammation

We and others (1–3) have noted that the pathological changes which characterize Alzheimer's disease (AD)[1] could all result from complement activation in neuritic plaques (NP), since this effector system has the ability to activate various cell types with release of cytokines and secondary mediators; to induce directed migration of these cells toward the complement activator; to alter cellular functions; and to damage cells (4, 5). Potential complement involvement in the brain is not dependent on disruption of the blood–brain barrier, since neurons, astrocytes, microglia, and oligodendrocytes synthesize most, and likely all, of the proteins of the complement system (6).

Since activation is a prerequisite for manifestation of all of the biological activities of the complement system, the β-amyloid peptide (Aβ) or another component of NP must possess the ability to activate complement in order for complement to be involved in mediating the pathologic cellular characteristics of AD. In this regard, we and others previously showed that fibrillar forms of Aβ bound the first reacting factor of the classical complement pathway (CCP), C1q (3, 7), and depleted the activity of the fourth complement component (C4) as well as whole complement activity (CH50), when incubated with human serum as a complement source in vitro (3, 7). Residues 14–26 of the collagen-like portion of the A polypeptide chain of the C1q molecule were implicated in binding fibrillar Aβ (7). Recent studies have confirmed this suggestive evidence of CCP activation by aggregated Aβ (8–12), and have also emphasized the critical role of the β-pleated structure of Aβ in mediating these effects (9, 11). Inhibition studies have implicated Aβ residues 1–11 in C1q binding (11, 12). The complement depletion, inhibition, and cleavage assays used in these various studies have provided suggestive evidence for CCP activation by fibrillar Aβ; however, as indirect assays, they are subject to other interpretations. In this context, we recently presented preliminary evidence suggesting that Aβ forms complexes with C3 after incubation of fibrillar Aβ with a complement source (9).

In the course of these studies, we found that the addition of fibrillar Aβ to a complement source led to the generation of covalent ester-linked complexes of Aβ with C3 activation fragments, providing unequivocal evidence for complement activation by Aβ, since covalent attachment

[1]*Abbreviations used in this paper:* ACP, alternative complement pathway; AD, Alzheimer's disease; Aβ, β-amyloid peptide; CCP, classical complement pathway; dd, double distilled; MAC, membrane attack complex; NP, neuritic plaques; NHS, normal human serum; SOD, superoxide dismutase.

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Volume 188, Number 3, August 3, 1998 431–438
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of C3 activation fragments to complement activators represents a fundamental tenet of complement action. We also found that fibrillar Aβ possesses the ability to activate the alternative complement pathway (ACP) in serum, as well as in mixtures of the six purified proteins of the alternative pathway in physiologic concentrations, providing the first indication that Aβ independently activates both complement pathways. Additionally, we observed that such activation is highly specific for Aβ and completely independent of oxidative processes. These studies are described here. Finally, we also report for the first time that Aβ-mediated complement activation is biologically significant, as it leads to generation of the cytokine-like C5a complement-activation fragment, and mediates formation of the proinflammatory C5b-9 membrane attack complex (MAC), in functionally active form able to insert into and permeabilize the membranes of neuronal precursor cells.

Materials and Methods

Aβ-Mediated Complement Activation and Complement Activation ELISA Assays. Aβ 1–40 and 1–42 (Bachem California, Torrance, CA; Bachem Bioscience, Inc., King of Prussia, PA; Quality Controlled Biochemicals, Inc., Hopkinton, MA; Anaspec, Inc., San Jose, CA; and California Peptide Research, Inc., Napa, CA) were dissolved in either 100% DMSO at 10 mg/ml, or double distilled (dd)H_2O, gradually diluted in ddH_2O to 2 mg/ml and then brought to 1 mg/ml in 0.1 M Tris buffer, pH 7.4. Aβ was used immediately (nonaggregated) or permitted to aggregate by incubation at room temperature for 48 h (Aβ 1–42) to 72 h (Aβ 1–40); further incubation for 2 wk did not decrease complement-activating potential. Preaggregated Aβ preparations were incubated with an equal volume of 1:5 normal human serum (NHS), complement-depleted sera (Advanced Research Technologies, Inc.), or the six purified proteins of the ACP (Advanced Research Technologies, Inc.) in physiologic ratios (13). Dilutions were in veronal buffered saline, pH 7.4, containing calcium and magnesium. Inhibition studies were carried out with preaggregated Aβ 1–42 in the presence of deferoxamine, glutathione, dimethylthiourea, catalase, or superoxide dismutase (SOD), all purchased from Sigma Chemical Co. (St. Louis, MO). After 1 h at 37°C, EDTA was added to stop further complement activation, and the samples were diluted (1:20–1:300) and added, in replicate, to microtitration wells precoated overnight (4°C) with 1 μg (100 μl) of mAb to Aβ (10D5), mAb to a C3b neoantigen (clone 129), or mAb to an iC3b neoantigen (Quidel, San Diego, CA) at pH 7.4, and then blocked (BLOTTO; Pierce Chemical Co., Rockford, IL). After 1 h at room temperature, bound Aβ–C3b/iC3b complexes were detected with rabbit Ab to C3 or to Aβ, horseradish peroxidase–conjugated anti–rabbit IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD), and ABTS (Kirkegaard & Perry Laboratories). C3 standard curves were generated with dilutions of purified iC3b (Advanced Research Technologies, Inc.) captured on wells precoated with anti-iC3b (Quidel). C5b-9 was captured on wells precoated with mAb to a C5b-9 neoantigen located in the poly C9 portion of the complex (Quidel) and detected with polyclonal goat Ab to C6 followed by horseradish peroxidase–conjugated mouse anti–goat IgG (Accurate Chemical and Science Corp., Westbury, NY) and ABTS. Values for the C5b-9 assay were back-calculated to the values in undiluted NHS. In some experiments, neuropeptide Y-porcine, urotensin I, or exendin 3 (all from California Peptide Research, Inc.); insulin B chain (Sigma Chemical Co.); amyloid precursor protein peptide (657–676 (Bachem California); and adenovirus penton base 50-residue fragment (residues 317–366; reference 14), as well as preaggregated Aβ 1–42 preparations and monomeric Aβ 1–42, were dissolved in DMSO or ddH_2O and aged at room temperature, exactly as described above for Aβ. In some studies, peptides, after dissolution as just described but diluted in Hepes-buffered NaCl at pH 7.4, were covalently cross-linked at a concentration of 200 μM with the primary amine reactive agent, bis(sulfosuccinimidyl)suberate (BS^3; Pierce Chemical Co.), at a concentration of 5 mM. After 30 min, the reaction was terminated by quenching. All peptides were incubated with NHS for 1 h at 37°C. After dilution, complement activation was assessed by the conventional CH50 assay (15) by quantitating residual functional C3 using C3-depleted serum according to product instructions (Advanced Research Technologies, Inc.), or by evaluating C5b-9 formation.

Studies with Hydroxylamine. After capture of complexes onto 10D5-coated wells, replicate wells were treated with 0.1 M Tris, pH 9.5, or 1 M hydroxylamine in 0.1 M Tris, pH 9.5, for 2 h at 37°C. After washing, remaining bound C3 was detected as described above. Residual Aβ was detected with rabbit Ab to Aβ, as described above. The formation of covalent complexes of Aβ with C3 activation products was also evaluated using the Western blotting procedure on SDS-PAGE gels. Replicate samples of aggregated Aβ 1–42 or Aβ 1–40 were incubated with human serum for 1 h at 37°C in the presence or absence of EDTA, and the reaction mixtures were then microfuged, washed in Tris buffer, and incubated for 3 h at 37°C with 0.1 M Tris at pH 7.4, 0.1 M Tris at pH 9.5, or 1 M hydroxylamine in 0.1 M Tris at pH 9.5. The samples were again microfuged, and washed with Tris at pH 7.4 followed by the same buffer containing 0.1% SDS. The samples were then subjected to SDS-PAGE under nonreducing conditions and electroblotted, and bands were detected with rabbit Ab to C3 followed by goat anti–rabbit IgG (Kirkegaard & Perry Laboratories) and the SuperSignal system (Pierce Chemical Co.). After stripping, Aβ was detected with 6E10 mouse Ab to Aβ (Senetek PLC, St. Louis, MO) followed by goat anti–mouse IgG (Kirkegaard & Perry Laboratories) and the SuperSignal system. In some experiments, electroblotted gels were reacted first with rabbit Ab to Aβ (generated in this laboratory) or with 6E10 mAb to Aβ (Senetek PLC) followed by goat anti–rabbit IgG (Kirkegaard & Perry Laboratories) or goat anti–mouse IgG (Kirkegaard & Perry Laboratories), stripped, and reacted with mAb anti-iC3b (Quidel) or rabbit Ab to C3 (generated in this laboratory), followed by goat anti–mouse IgG or goat anti–rabbit IgG. Quantitation was with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Mass Spectroscopy. After solubilization in 70% formic acid, samples were analyzed by MALDI spectroscopy (Perseptive Voyager ELITE; Perseptive Biosystems, Inc., Framingham, MA).

C5a. C5a (and C5a des-Arg) was detected in diluted samples with the Biotrak radioimmunoassay kit (Amersham Corp., Arlington Heights, IL); the samples were subjected to acid precipitation before analysis (16). Values were back-calculated to the concentrations in undiluted NHS.

C5b-9 Membrane Insertion. Ntera2/D1 (NT2) cells (Stratagene Inc., La Jolla, CA) were grown to subconfluence, released with nonenzymatic cell dissociation solution (GIBCO BRL, Gaithersburg, MD), washed, and resuspended (2×10^7 cells/ml). NT2 cells (100 μl) were incubated with 50 μl NHS in the presence or absence of EDTA and 50 μl preaggregated Aβ. After 15 min at 37°C, the MAC was detected with rabbit Ab (Advanced Re-

search Technologies, Inc.) or mAb (Quidel) to C5b-9 neoantigens, followed by FITC anti-rabbit or -mouse Ig and propidium iodide. Readings were performed on a FACScan® and analyzed with CellQuest software (Becton Dickinson, San Jose, CA).

Results and Discussion

Sandwich-type ELISAs showed that complexes containing Aβ and C3b/iC3b were generated in NHS, as a complement source, after incubation with aggregated Aβ 1–42. Complexes were demonstrable after capture with mAbs to activation-dependent neoantigens in the first (C3b) or second (iC3b) C3 cleavage products and detection with rabbit Ab to Aβ (Fig. 1, *a* and *b*), as well as after capture with mAb to Aβ and detection with rabbit Ab to C3 (Fig. 1 *c*) or C3d (not shown). EDTA, which blocks complement activation by chelating calcium and magnesium, prevented complex formation (Fig. 1 *c*). ELISAs in which complexes were captured with mAb to Aβ and detected with Ab to C3 were used for most of the studies, since such ELISAs permitted quantitation by reference to included standard curves generated with purified C3 captured on wells coated with mAb to C3 and detected with rabbit Ab to C3 (Fig. 1 *d*). Complement activation was detectable to ~1 μM Aβ 1–42 (Fig. 1 *d*). 10 different preaggregated Aβ 1–40 and 20 different preaggregated Aβ 1–42 preparations from 5 manufacturers generated such complexes. Aβ 1–42 was generally 5–10-fold more active than Aβ 1–40 in this regard. The cation-dependent formation of Aβ–C3b/iC3b complexes after incubation of aggregated Aβ with NHS provides unequivocal evidence for complement activation by aggregated Aβ.

Aβ–C3b/iC3b complex formation was evident after incubation of aggregated Aβ 1–42 with NHS lacking factor B, an essential component of the ACP (Fig. 1 *e*); such sera contain an intact CCP, but do not permit ACP activation. A significant reduction in complex formation was also evident in C1q-depleted serum compared with NHS (Fig. 1 *e*). These data show that the CCP mediates complex formation by aggregated Aβ, findings that were anticipated from the results of the complement depletion assays described earlier. Unexpectedly, however, the ACP also mediated the formation of complexes, since they were also generated after the addition of aggregated Aβ 1–42 to NHS lacking factor C1q, and complex formation was reduced in factor B–depleted serum compared with NHS (Fig. 1 *e*), a result replicated in four additional experiments with different Aβ 1–42 preparations. The ability of Aβ 1–42 to activate the ACP was confirmed in three studies in which preaggregated Aβ 1–42 was incubated with a mixture of the six purified proteins of the ACP (factors B, D, H, and I, properdin, and C3) in physiological ratios (reference 13; Fig. 1 *f*). These data document the ability of aggregated Aβ not only to activate the CCP, but also to independently activate the ACP. This is the first indication that aggregated Aβ activates the ACP; it had been presumed that complement activation by Aβ was exclusively via the CCP because of the absence of ACP components (factor B and properdin) in NP (1, 17, 18). The failure to detect ACP components in NP may be due to the extreme lability of the ACP C3 convertase.

Multiple different aggregated Aβ 1–42 preparations activated complement, as determined by the classical CH50 complement consumption technique (Fig. 1 *g*). The aging

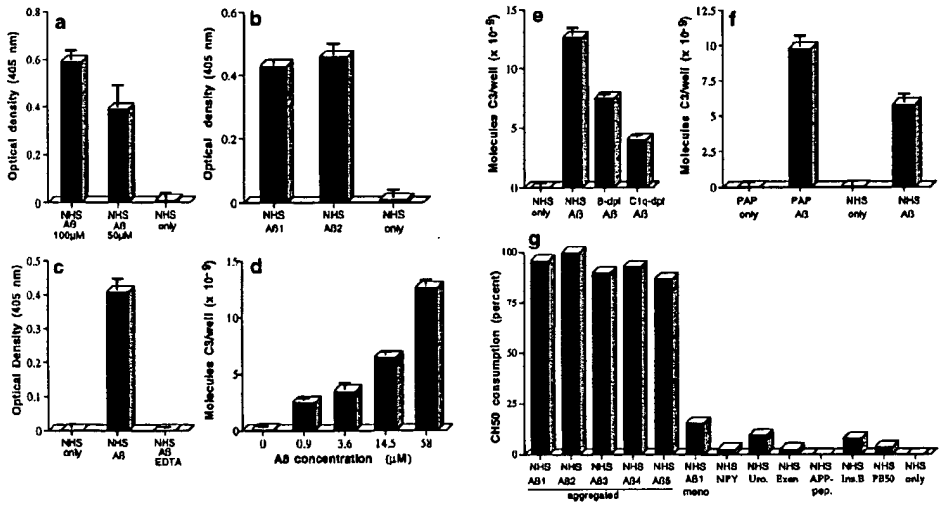

Figure 1. ELISA demonstration of complement-mediated formation of complexes of Aβ with C3 activation fragments (*a–d*). Complexes were captured, detected, and quantitated as described in Materials and Methods. NHS or purified ACP proteins only do not contain Aβ. (*a*) Preaggregated Aβ 1–42 (100 μM and 50 μM) was incubated in NHS, captured with mAb to C3b, and detected with rabbit Ab to Aβ. (*b*) Two preaggregated Aβ 1–42 preparations (Aβ 1 and Aβ 2, at 20 μM) were incubated in NHS, captured with mAb to iC3b, and detected with rabbit Ab to Aβ. (*c*) Preaggregated Aβ 1–42 (58 μM) was incubated in NHS, or in NHS containing 10 mM EDTA, captured with mAb to Aβ, and detected with rabbit Ab to C3. (*d*) Preaggregated Aβ 1–42 was incubated in NHS at the indicated final concentrations, captured with mAb to Aβ, and detected with rabbit Ab to C3. (*e*) Preaggregated Aβ 1–42 (58 μM) was incubated in NHS, factor B–depleted NHS (*B-dpl*), or C1q-depleted NHS (*C1q-dpl*), captured with mAb to Aβ, and detected with rabbit Ab to C3. (*f*) Preaggregated Aβ 1–42 (58 μM) was incubated with the six purified ACP proteins (*PAP*) or NHS, captured with mAb to Aβ, and detected with rabbit Ab to C3. Background levels obtained in EDTA controls containing Aβ and purified ACP proteins or NHS, in the various experiments described above, were subtracted. (*g*) Specificity of complement activation. Preaggregated Aβ preparations (20 μM) and the same concentrations of monomeric Aβ (*mono*), insulin B chain (*Ins.B*), neuropeptide Y-porcine (*NPY*), urotensin I (*Uro.*), exendin 3 (*Exen*), amyloid precursor peptide 657–676 (*APP-pep.*), and adenovirus penton base 50-residue peptide (*PB50*) were incubated with NHS. Complement activation was assessed by the CH50 method. Correlation coefficients for the CH50 determinations ranged from 0.995 to 1.000.

procedure used to aggregate Aβ generates β-pleated fibrils (9). Nonfibrillar "amorphous" aggregates of Aβ are devoid of complement-activating ability (9). In contrast to the aggregated preparations, Aβ used immediately after dissolution had limited ability to activate complement (Fig. 1 *g*). These data document the important role of fibril formation for complement activation by Aβ in vitro. Amylin, another peptide which spontaneously forms β-pleated fibrils, was also tested for complement-activating ability in these studies. The 37-residue amylin polypeptide represents the principal constituent of the amyloid deposits in type 2 diabetes. On SDS-PAGE gels, aged amylin migrated primarily as large SDS-insoluble stained bands. However, this fibrillar peptide did not significantly activate complement at a concentration of 100 μM (7% CH50 consumption).

The specificity of complement activation by Aβ was also evaluated by determining whether complement was activated by other small peptides (20–50 amino acids) containing multiple residues able to mediate covalent linkage to the glutamate residue of the hydrolyzed thioester of C3 (serine, tyrosine, threonine, lysine) and expressing similar overall charge to Aβ. All of the peptides were processed and aged in the same manner as Aβ. None of the peptides, including the insulin B chain (30 residues), neuropeptide Y-porcine (36 residues), urotensin I (41 residues), exendin 3 (39 residues), amyloid precursor peptide 657–676 (20 residues), and the adenovirus penton base fragment (50 residues) significantly activated complement at a concentration of 20 μM, as assessed by the classical CH50 technique (Fig. 1 *g*). The peptides also showed little or no ability to activate complement in other assays, including the ability to deplete residual functional C3 and form the SC5b-9 complex (not shown). To determine whether peptide aggregation would increase complement-activating ability, urotensin I, neuropeptide Y-porcine, and exendin 3 were covalently cross-linked with the primary amine reactive reagent BS[3] before evaluating their ability to activate complement by the CH50 technique. Cross-linked urotensin I and neuropeptide Y-porcine gave a ladder of Coomassie-stained bands on SDS-PAGE analyses, but exendin 3 gave no stained bands, possibly due to the formation of very large aggregates. These three cross-linked peptides did not significantly activate complement (<10% CH50 depletion) at a concentration of 20 μM. In another study, cross-linked urotensin I exhibited 7% CH50 consumption at a concentration of 100 μM, whereas aggregated Aβ 1–42 showed 45% consumption. These data cumulatively demonstrate the marked specificity of complement activation by fibrillar Aβ.

C3 preferentially binds to activators via ester bonds, although amide linkage has been described (19, 20); such bonds form between the reactive γ-carbonyl group of the glutamate residue of the activation-cleaved internal thioester bond in C3, and hydroxyl (ester) or amino (amide) groups on the activator (19). To evaluate possible ester linkage, complexes were captured with mAb to Aβ and incubated with Tris buffer containing 1 M hydroxylamine at pH 9.5 for 2 h at 37°C, a treatment which disrupts ester but not amide bonds (19, 20). Approximately 50% of the bound C3, but none of the Aβ, was removed from the captured complexes by this treatment (Fig. 2, *a* and *b*), a result replicated in two additional studies with Aβ 1–40 and 1–42.

The formation of covalent complexes of Aβ with C3 activation products was also independently demonstrated using a Western blotting approach. In these studies, aggregated Aβ 1–40 was incubated with serum, and the complexes of insoluble fibrillar Aβ with C3 activation fragments were then sedimented, washed, and incubated with either 1 M hydroxylamine at pH 9.5 or control buffers. After washing, a prominent band with a molecular mass of ~180 kD, the molecular mass of C3, as well as several higher molecular mass bands, were detected with Ab to C3 (Fig. 2 *c*). After stripping, the same bands were also found to react with Ab to Aβ, although the gels were darker due to the presence of large amounts of aggregated Aβ (Fig. 2 *c*). Bands of the same molecular masses reactive with Abs to both Aβ and C3 were also observed when the blotting studies were performed in the reverse direction, i.e., blotting first with either mAb or polyclonal Ab to Aβ followed, after stripping, by blotting with rabbit Ab or mAb to C3 (not shown). The 180-kD band and the larger bands, which contain both C3 and Aβ, undoubtedly represent complexes of Aβ monomers with C3b monomers and oligomers, since they were not evident in the reactions carried out in the presence of EDTA or in the absence of fibrillar Aβ (Fig. 2 *c*).

C3 was also detected in the large Aβ aggregates on the top of the gels (except for the EDTA lane) on longer exposure (not shown). The lesser reactivity of C3 in the larger Aβ aggregates at the top of the gels, compared with the C3 monomers and oligomers within the gels, indicates that not all Aβ monomers bear a molecule of C3b; this is not surprising, since Aβ is in large aggregates and, in addition, in molar excess over C3. It may also be that Aβ molecules bearing covalently bound C3b dissociate from the aggregates, in analogy to the dissociation of immune complexes by the covalent binding of C3b (21, 22).

Hydroxylamine treatment disrupted approximately half of the complexes (Fig. 2 *c*). Quantitative scanning of the C3 Western blot showed that the treatment with 1 M hydroxylamine at pH 9.5 removed 42% of the bound C3 compared with the pH 9.5 control; the pH 9.5 buffer treatment removed only trivial amounts (5.1%) of the bound C3 compared with the pH 7.4 treatment. The Aβ Western blot could not be satisfactorily scanned due to the large background, but visual inspection reveals the same pattern (Fig. 2 *c*). Identical results were obtained with Aβ 1–42 (not shown). These two independent assay systems both show that ester bonds, in part, mediate covalent attachment of C3 activation fragments to Aβ. Aβ 1–42 contains two serines, at positions 8 and 26, and a tyrosine, at position 10, which could mediate ester linkages with C3 activation fragments.

With regard to the bond(s) responsible for the nonester-linked Aβ–C3b/iC3b complexes, Aβ alone has been reported to generate free radicals (23) upon incubation in aqueous solution, and oxidative processes have been associated with Aβ denaturation, fragmentation, and oxidation (24, 25). Because of the potential relevance of these pro-

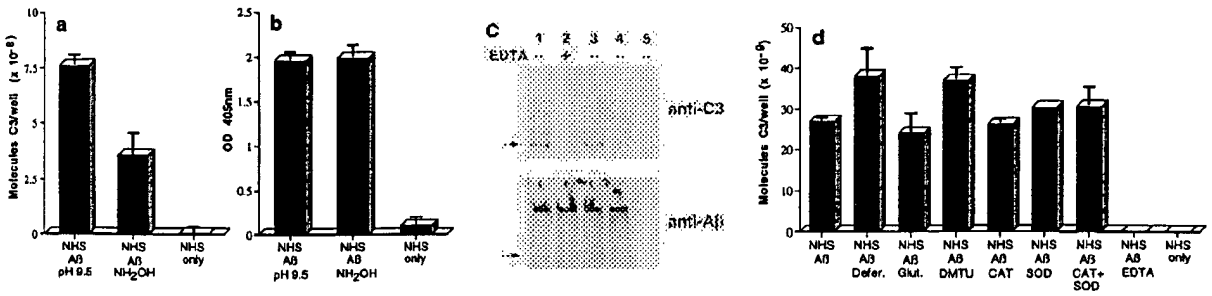

Figure 2. Assessment of bonds mediating binding of Aβ to C3 activation fragments. (*a*) Preaggregated Aβ 1–42 (25 μM) was incubated in NHS, and complexes were captured on 10D5-coated wells; NHS only does not contain Aβ. Replicate samples were treated with pH 9.5 buffer, or with the same buffer containing 1 M hydroxylamine (NH_2OH), and remaining bound C3 was then detected and quantitated as described in Materials and Methods. The control containing Aβ, NHS, and EDTA has been subtracted. (*b*) Replicate wells subjected to treatment with the pH 9.5 buffer or hydroxylamine were evaluated for residual bound Aβ as described in Materials and Methods. (*c*) Preaggregated Aβ 1–40 was incubated in NHS in the presence or absence of EDTA; lane *5* contains NHS but no Aβ. After centrifugation and washing, fibrillar Aβ pellets were incubated with pH 7.4 buffer (lanes *1* and *2*), pH 9.5 buffer (lane *3*), or 1 M hydroxylamine in pH 9.5 buffer (lane *4*). After further washing, samples were subjected to SDS-PAGE under nonreducing conditions followed by blotting for the presence of C3 and, after stripping, for Aβ. *Arrow*, The C3 band at ~180 kD. (*d*) Preaggregated Aβ 1–42 (50 μM) was incubated in NHS alone, and in the presence of deferoxamine (*Defer.*; 1 mM), glutathione (*Glut.*; 1 mM), dimethylthiourea (*DMTU*; 30 mM), catalase (*CAT*; 2×10^4 U/ml), SOD (10 μM), or catalase plus SOD, and the Aβ complexes with C3 activation fragments were then detected as described in Materials and Methods.

cesses to the formation of complexes of Aβ with C3 activation fragments, Aβ 1–42 was assessed by MALDI mass spectroscopy after aging from 0 to 10 d. Aggregates are not detected in these assays, since the samples are dissolved in 70% formic acid for mass spectroscopic analysis. The molecular mass of the major peak in the various samples ranged from 4510 to 4514, and no other peaks were present, ruling out significant oxidation, fragmentation, and covalent cross-linking of Aβ. To determine whether oxidative processes mediated the formation of complexes of Aβ with C3 activation fragments, complement activation was carried out in the presence of deferoxamine, glutathione, dimethylthiourea, catalase, SOD, and catalase plus SOD. Since none of these antioxidants or free radical scavengers inhibited the formation of or interfered with the detection of complexes (Fig. 2 *d*), it is unlikely that free radical–mediated or oxidative processes are involved in the formation of complexes of Aβ with C3 activation fragments. In all likelihood, amide bonds are responsible for the remaining Aβ–C3b/iC3b complexes. Aβ 1–42 contains two lysine residues, at positions 16 and 28, which could mediate such linkages.

Additional studies showed that Aβ triggered activation of the terminal, proinflammatory portion of the complement-reaction sequence in NHS. C5a, a cytokine-like activation cleavage product of C5 with numerous biological properties, was efficiently generated by aggregated Aβ 1–42 in NHS, as determined by a specific radioimmunoassay which detects C5a and C5a des-Arg (lacking the COOH-terminal arginine residue) (reference 26; Fig. 3 *a*). A sandwich ELISA in which an mAb to a C5b-9 neoantigen located in poly C9 served as the capture Ab, and polyclonal Ab to C6 served as the detection Ab, showed that Aβ-mediated complement activation led to formation of the C5b-9 complex (Fig. 3 *b*). This ELISA detects C5b-9 as well as SC5b-9 complexes; the latter are formed in NHS in the absence of cells, as a consequence of the binding of S protein, a complement control protein, to the complex. Aβ 1–42 was generally more efficient in generating C5b-9 than Aβ 1–40 (Fig. 3 *b*). In contrast, another group recently reported that Aβ-mediated complement activation does not lead to generation of the C5b-9 complex (10). The reason(s) for their failure to demonstrate C5b-9 formation is not known. One possibility is the well-known variability in the properties of different Aβ preparations. In this regard, we have observed more variability in the ability of various Aβ 1–40 and Aβ 1–42 preparations to trigger C5b-9 complex formation, than in their ability to generate Aβ–C3b and Aβ–iC3b complexes. Other explanations could lie in slight differences in experimental conditions. For example, their C5b-9 formation experiments were carried out in NHS diluted 1:10 in phosphate-buffered NaCl; this combination provides suboptimal concentrations of calcium and magnesium, which are required for CCP and ACP activation. In this regard, we obtained 10-fold higher levels of SC5b-9 formation than they obtained with 1 μM aggregated IgG in their control studies (not shown).

The C5b-9 complex generated by Aβ 1–42-mediated complement activation was able to insert into the membranes of NT2 cells, a committed neuronal precursor cell line, when such cells were included in reaction mixtures with aggregated Aβ and NHS (Fig. 4 *a*). Depicted are flow cytometric analyses with rabbit Ab to activation-specific neoantigens in the C5b-9 MAC. C5b-9 membrane insertion was likely proportional to the extent of complement activation, since it was dependent on the concentration of Aβ 1–42. mAb to C5b-9 neoantigens gave the same result (not shown). Identical Aβ 1–40 concentrations mediated lower levels of C5b-9 membrane insertion (not shown), probably because of the significantly lower levels of C5b-9 formation with Aβ 1–40. NT2 cells and other neuronal cell lines are resistant to complement-dependent cytolysis,

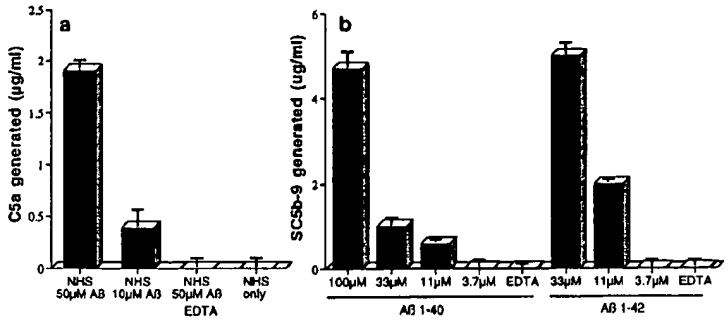

Figure 3. Aβ-mediated complement activation generates C5a and the MAC. (*a*) Preaggregated Aβ 1–42 was incubated in NHS, and C5a generation was then quantitated as described in Materials and Methods. (*b*) C5b-9 was quantitated after NHS was incubated with varying concentrations of preaggregated Aβ 1–42 or 1–40. SC5b-9 formation was quantitated as described in Materials and Methods.

likely because of the presence of CD59 (27), a complement regulatory protein, a finding confirmed here. Nevertheless, C5b-9 insertion into NT2 cell membranes mediated an increase in the permeability of the cells to propidium iodide that was dependent on the concentration of Aβ 1–42 (Fig. 4 *b*). These data indicate that C5b-9 generated by Aβ-mediated complement activation is functionally competent, since it inserts into the membranes of neuronal precursor cells and renders them permeable to small molecules.

Thus, Aβ directly and independently activates the ACP as well as the CCP, leading to the formation of covalent Aβ–C3b and Aβ–iC3b complexes; generates C5a; and mediates assembly of functionally active C5b-9 complexes in vitro. These findings have potential implications for understanding the mechanisms which lead to continuing neuronal damage and altered glial functions in the vicinity of NP, and thus to the progression of AD. First, they provide an explanation for the association of bound C3 with Aβ in NP (1–3), since covalently bound C3b molecules in NP would remain bound and provide a nidus for chronic complement activation. Second, C5a generated by Aβ-mediated complement activation could be responsible for the increased numbers of activated astrocytes and microglia around NP compared with diffuse Aβ plaques (28), since these cells possess C5a receptors and are activated and migrate in response to C5a (6, 29–31). C5a could also trigger the release of proinflammatory cytokines (IL-1, IL-6, IL-8, and TNF-α) from glial cells, as it does from other cell types (26, 32); proinflammatory cytokines are increased in the AD brain (2, 28, 33). These cytokines could further activate glial cells and alter neuronal and glial functions (28, 32). Third, incoming activated glial cells could bind and remain adherent, via their complement receptors, to C3 activation fragments attached to Aβ (6). Fourth, C5b-9 insertion into cell membranes provides an explanation for the association of this complex with dystrophic neurites in NP (2, 3). Although not likely to be directly cytotoxic for neurons, since they bear CD59 (6, 34), C5b-9 as well as C5b-7 and C5b-8 complexes could alter neuronal functional properties over time by chronic low-level triggering of various cellular signaling pathways (35). If this inflammation-based scenario is verified, complement inhibitors should be evaluated for use in AD. Such inhibitors would need to pass the blood–brain barrier, target both complement activation pathways, and prevent C5b-9 activation.

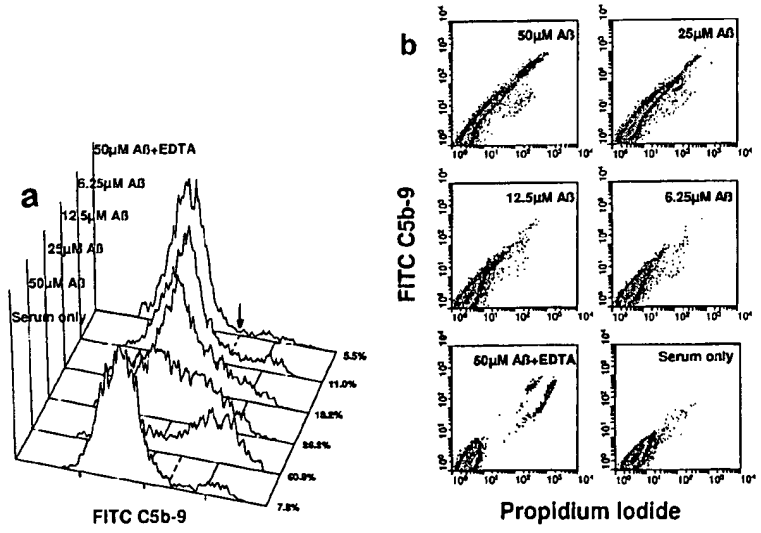

Figure 4. C5b-9 generated by Aβ 1–42-mediated complement activation is functionally active. (*a*) NT2 cells were incubated with preaggregated Aβ 1–42 at the designated concentrations in NHS, or in NHS containing 10 mM EDTA; serum only lacks Aβ. Flow cytometric analyses with a rabbit Ab to C5b-9 neoantigens are shown. *Numbers* (*right*), Percentage of C5b-9$^+$ cells, as determined by their relationship to the marker (*arrow, dashed line*). (*b*) Density plot analyses of propidium iodide and C5b-9 (*FITC C5b-9*) reactivities are shown. For clarity, only live cells are depicted.

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References

1. Eikelenboom, P., and F.C. Stam. 1982. Immunoglobulins and complement factors in senile plaques. *Acta Neuropathol.* 57:239–242.
2. McGeer, P.L., and E.G. McGeer. 1995. The inflammatory response system of brain: implications for therapy of Alzheimer and other neurodegenerative diseases. *Brain Res. Brain Res. Rev.* 21:195–218.
3. Rogers, J., N.R. Cooper, S. Webster, J. Schultz, P.L. McGeer, S.D. Styren, W.H. Civin, L. Brachova, B. Bradt, P. Ward, and I. Lieberburg. 1992. Complement activation by β-amyloid in Alzheimer disease. *Proc. Natl. Acad. Sci. USA.* 89:10016–10020.
4. Müller-Eberhard, H.J. 1988. Molecular organization and function of the complement system. *Annu. Rev. Biochem.* 57: 321–347.
5. Cooper, N.R. 1998. Biology of the complement system. *In* Inflammation: Basic Principles and Clinical Correlates. J. Gallin and R. Snyderman, editors. Lippincott-Raven Publishers, Philadelphia. In press.
6. Morgan, B.P., and P. Gasque. 1996. Expression of complement in the brain: role in health and disease. *Immunol. Today.* 17:461–466.
7. Jiang, H., D. Burdick, C.G. Glabe, C.W. Cotman, and A.J. Tenner. 1994. β-Amyloid activates complement by binding to a specific region of the collagen-like domain of the C1q A chain. *J. Immunol.* 152:5050–5059.
8. Chen, S., R.C.A. Frederickson, and K.R. Brunden. 1996. Neuroglial-mediated immunoinflammatory responses in Alzheimer's disease: complement activation and therapeutic approaches. *Neurobiol. Aging.* 17:781–787.
9. Webster, S., B. Bradt, J. Rogers, and N.R. Cooper. 1997. Aggregation state-dependent activation of the classical complement pathway by the amyloid β peptide (Aβ). *J. Neurochem.* 69:388–398.
10. Cadman, E.D., and P.S. Puttfarcken. 1997. β-Amyloid peptides initiate the complement cascade without producing a comparable effect on the terminal pathway *in vitro*. *Exp. Neurol.* 146:388–394.
11. Webster, S., B. Bonnell, and J. Rogers. 1997. Charge-based binding of complement component C1q to the Alzheimer amyloid β-peptide. *Am. J. Pathol.* 150:1531–1536.
12. Velazquez, P., D.H. Cribbs, T.L. Poulos, and A.J. Tenner. 1997. Aspartate residue 7 in amyloid β-protein is critical for classical complement pathway activation: implications for Alzheimer's disease pathogenesis. *Nat. Med.* 3:77–79.
13. Schreiber, R.D., M.K. Pangburn, P.H. Lesavre, and H.J. Müller-Eberhard. 1978. Initiation of the alternative pathway of complement: recognition of activators by bound C3b and assembly of the entire pathway from six isolated proteins. *Proc. Natl. Acad. Sci. USA.* 75:3948–3952.
14. Wickham, T.J., P. Mathias, D.A. Cheresh, and G.R. Nemerow. 1993. Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ promote adenovirus internalization but not virus attachment. *Cell.* 73:309–319.
15. Mayer, M.M. 1961. Complement and complement fixation. *In* Experimental Immunochemistry. E.A. Kabat and M.M. Mayer, editors. Charles C. Thomas Publisher, Springfield, IL. 133–241.
16. Wagner, J.L., and T.E. Hugli. 1984. Radioimmunoassay for anaphylatoxins: a sensitive method for determining complement activation products in biological fluids. *Anal. Biochem.* 136:75–88.
17. McGeer, P.L., H. Akiyama, S. Itagaki, and E.G. McGeer. 1989. Immune system response in Alzheimer's disease. *Can. J. Neurol. Sci.* 16:516–527.
18. McGeer, P.L., H. Akiyama, S. Itagaki, and E.G. McGeer. 1989. Activation of the classical complement pathway in brain tissue of Alzheimer patients. *Neurosci. Lett.* 107:341–346.
19. Law, S.K.A., N.A. Lichtenberg, and R.P. Levine. 1979. Evidence for an ester linkage between the labile binding site of C3b and receptive surfaces. *J. Immunol.* 123:1388–1394.
20. Gadd, K.J., and K.B.M. Reid. 1981. The binding of complement component C3 to antibody-antigen aggregates after activation of the alternative pathway in human serum. *Biochem. J.* 195:471–480.
21. Miller, G.W., and V. Nussenzweig. 1975. A new complement function: solubilization of antigen-antibody aggregates. *Proc. Natl. Acad. Sci. USA.* 72:418–422.
22. Fujita, T., Y. Takata, and N. Tamura. 1981. Solubilization of immune precipitates by six isolated alternative pathway proteins. *J. Exp. Med.* 154:1743–1751.
23. Harris, M.E., K. Hensley, D.A. Butterfield, R.A. Leedle, and J.M. Carney. 1995. Direct evidence of oxidative injury produced by the Alzheimer's β-amyloid peptide (1-40) in cultured hippocampal neurons. *Exp. Neurol.* 131:193–202.
24. Dyrks, T., E. Dyrks, T. Hartmann, C. Masters, and K. Beyreuther. 1992. Amyloidogenicity of βA4 and βA4-bearing amyloid protein precursor fragments by metal-catalyzed oxidation. *J. Biol. Chem.* 267:18210–18217.
25. Hensley, K., D.A. Butterfield, N. Hall, P. Cole, R. Subramanian, R. Mark, M.P. Mattson, W.R. Markesbery, M.E. Harris, M. Aksenov, et al. 1996. Reactive oxygen species as causal agents in the neurotoxicity of the Alzheimer's disease-associated amyloid beta peptide. *Ann. NY Acad. Sci.* 786: 120–134.
26. Hugli, T.E. 1984. Structure and function of the anaphylatoxins. *Springer Semin. Immunopathol.* 7:193–219.

27. Shen, Y., J.A. Halperin, and C.-M. Lee. 1995. Complement-mediated neurotoxicity is regulated by homologous restriction. *Brain Res.* 671:282–292.
28. Griffin, W.S.T., J.G. Sheng, G.W. Roberts, and R.E. Mrak. 1995. Interleukin-1 expression in different plaque types in Alzheimer's disease: significance in plaque evolution. *J. Neuropathol. Exp. Neurol.* 54:276–281.
29. Yao, J., L. Harvath, D.L. Gilbert, and C.A. Colton. 1990. Chemotaxis by CNS macrophage, the microglia. *J. Neurosci. Res.* 27:36–42.
30. Lacy, M., J. Jones, S.R. Whittemore, D.L. Haviland, R.A. Wetsel, and S.R. Barnum. 1995. Expression of the receptors for the C5a anaphylatoxin, interleukin-8 and FMLP by human astrocytes and microglia. *J. Neuroimmunol.* 61:71–78.
31. Ilschner, S., C. Nolte, and H. Kettenmann. 1996. Complement factor C5a and epidermal growth factor trigger the activation of outward potassium currents in cultured murine microglia. *Neuroscience.* 73:1109–1120.
32. Benveniste, E.N. 1992. Inflammatory cytokines within the central nervous system: sources, function, and mechanism of action. *Am. J. Physiol.* 263:C1–C16.
33. Selkoe, D.J. 1994. Alzheimer's disease: a central role for amyloid. *J. Neuropathol. Exp. Neurol.* 53:438–447.
34. Vedeler, C., E. Ulvestad, L. Bjorge, G. Conti, K. Williams, S. Mork, and R. Matre. 1994. The expression of CD59 in normal human nervous tissue. *Immunology.* 82:542–547.
35. Rus, H.G., F. Niculescu, and M.L. Shin. 1996. Sublytic complement attack induces cell cycle in oligodendrocytes. *J. Immunol.* 156:4892–4900.